

Optical Immune Biosensors for *Salmonella* Typhimurium Detection

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Abstract

In this work, high-specific biosensors for *Salmonella typhimurium* detection has been designed based on the surface plasmon resonance (SPR) and total internal reflection ellipsometry (TIRE) through high sensitivity and simplicity of analysis have been demonstrated at the same time. First as a registering part for our experiments Spreeta SPR device (USA) was applied and then the SPR biosensor with flowing cell named as "Plasmonotest" was used for the same aims. Previous researches confirmed an efficiency of SPR biosensors used to detect specific antigen-antibody interactions therefore this type of reactions with some previous preparations of surface binding layer was used as reactive part. It has been defined that in case with Spreeta sensitivity was on the level $10^3 - 10^7$ cells/ml. Another biosensor based on the SPR has shown the sensitivity within $10^1 - 10^6$ cells/ml. Maximal sensitivity was on the level of several cells (less than 5) in 10 ml which has been obtained using the biosensor based on TIRE.

Keywords

Zoonotic Infections; Antigen-antibody Reaction; Surface Plasmon Resonance; Total Internal Reflection Ellipsometry; *Salmonella Typhimurium*

Introduction

Salmonella spp. is one of the most frequently occurring food borne pathogens affecting the microbial safety of food and causes great concern in the food industry. Representatives of this species can cause different pathogenic diseases which provide a harmful influence to all body systems and are hardly treated. Majority of *Salmonella* is pathogenic for human and animals but in epidemiological meaning only a few of them have especially significant part. Infection spreads very fast and can afflict large quantity of people or animals. For example, it is estimated that about 1,4 million *Salmonella* infections occur each year in the United States, and cause more than 500 deaths annually [Valadez et al., 2009]. Another big concern is

Salmonella spp. antibiotic resistance. Among 180 raw food samples, including chicken, beef, pork, and shellfish samples about half (50.5%) of the isolates is resistant to at least one antibiotic. Furthermore, from all food types are isolated multiresistant *Salmonella* isolates resistant to at least three different classes of antibiotics [Van et al., 2007]. This fact complicates treatment of diseases caused by *Salmonella* representatives, and makes it possible to use resistant *Salmonella spp.* as a biological weapon [Shah et al., 2003; Shah et al., 2003; www.globalsecurity.org].

Unfortunately, traditional methods of *Salmonella spp.* isolating and identifying consist of a multi-step process that involves preenrichment, selective enrichment, biochemical testing and serological confirmation [Pividori et al., 2003]. These cultural techniques for detection of *Salmonella spp.* require 3–4 days to provide presumptive results and additional 1–2 days for further biochemical confirmation. As a rule traditional approaches used to reveal infected organisms are time-consuming, routine and demand special laboratory equipment with professional staff.

As alternative methods for *Salmonella* detection, Enzyme-linked immunosorbent assay (ELISA) and different tips of Polymerase Chain Reaction (PCR) are widely used. However, these methods also have a row of disadvantages. For ELISA, first of all, there are high limits of sensitivity greater than 10^5 cfu/ml [Cox, 1988], cross reactivity [Westerman et al., 1997], and changes to antigens due to acetylation and changing recognition by assay antibodies [Kim et al., 1999]. PCR methods, although sensitive and specific under optimized conditions, are time consuming and labour intensive due to post-amplification steps, not sensitive enough to measure the accumulated DNA copies accurately, and can only provide a qualitative result. Furthermore, they need several preenrichment steps that are routine and time-consuming.

Reliability and diagnostic significance of laboratory methods can be improved using instrumental analytical devices based on the principles of biosensorics. There is a wide spectrum of biosensors according to the main principles they are based on. This article is devoted to optical biosensors especially to their sensitivity which depends strongly on previous transducer preparation and optical module working characteristics. In our case, we tried to work out high-specific biosensors for *Salmonella typhimurium* detection based on the surface plasmon resonance (SPR) and total internal reflection ellipsometry (TIRE). Recently, these devices have been successfully used for the diagnostics of bovine retroviral leucosis [Nabok et al., 2005], and quantitative estimation of the presence of some mycotoxins among environmental objects [Nabok et al., 2007, 2010]. High sensitivity and simplicity of analysis have been demonstrated at the same time.

As a registering part for our researches, the devices based on the surface plasmon resonance and total internal reflection ellipsometry were used. First experiments were carried out using Spreeta biosensor (TI, USA) and the SPR device with flowing cell named as "Plasmonotest" (property of V.M. Glushkov Institute of Cybernetics of National Academy of Sciences, Ukraine; pat. UA 100934). TIRE based biosensor was used next for the same aims.

Previous researches confirmed an efficiency of SPR biosensors used to detect specific antigen-antibody interactions [Pirogova et al., 2002]. Therefore, this type of reactions with some previous preparations of surface binding layer was used as reactive part. The procedure of transducer preparation included several sequential steps: a) covering of surface by polyallylamine hydrochloride b) immobilization of protein A from *Staphylococcus aureus* and, at last, the oriented binding of the specific antibodies.

Materials and methods

Optical Systems Variants

Surface plasmon resonance (SPR) is one of the optical techniques for microorganisms detection widely applied for biosensors development. SPR-based instruments use an optical method to measure the refractive index (within ~300 nm) near a sensor surface which is presented as thin (about 50 nm) metal usually gold film [Oh et al., 2004; Son et al., 2007; Syam et al., 2012]. SPR-based devices operate as a flow systems

and have a flowing cell where liquids are pumped permanently. The angle position of SPR minimum is determined by the properties of the gold-solution interface. In addition, adsorption phenomena and kinetics can be monitored using SPR technique. Antigen-antibody reaction is the most widely used biological sensing element of transducer preparation for SPR biosensors. SPR has successfully been applied for pathogen bacteria detection by means of immunoreactions.

First Spreeta-based biosensor was used for pathogen detection [Son et al., 2007; Starodub et al., 2010; 2011]. Principle of "Plasmonotest" working is very close to Spreeta module organization. It is optical device of angular type based on the SPR principle, which is equipped with CCD array of 2048 pixels (Fig.1).

The software for information processing and data acquisition have been developed in the Institute of Cybernetics [Budnyk et al., 2011]. Angular resolution of device is 0.001 degree. Additionally, the flowing cell for liquor pumping and sample temperature stabilization could be mounted. Accuracy of temperature stabilization in flow cell is at least 0.1°C.

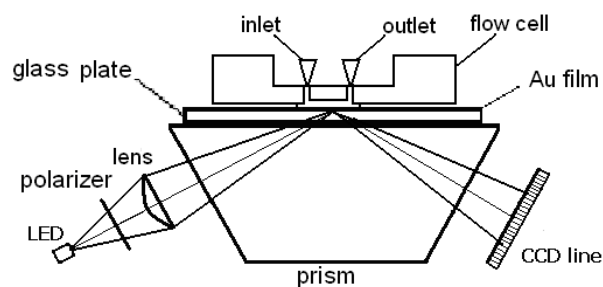


FIG. 1 SCHEME OF "PLASMONOTEST" DEVICE WITH FLOW CELL

One pronounced difference is that detecting layer is formed on the glass plate surface covered by 1-2nm niobium adhesive film and 50nm gold plasmon supporting film connected with prism using immersion liquid. This is more favorable because glass plates can be easily changed or/and renewed. Furthermore, it allows providing of all surface preparations in advance.

As a basis for TIRE biosensor, a commercial spectroscopic rotating analyzer instrument was used operated in the 350–1000 nm wave-length range. Detecting layer was formed on the chromium/gold-coated glass plate attached to a 68 trapezoidal glass prism (BK7, $n = 1.515$). This prism provides total internal reflection effect between glass and aqueous solutions ($n = 1.33$). The samples analysis was provided using a specially designed 1.5 cm³ cell attached through a rubber ring.

The injection of different solutions into the cell was carried with inlet and outlet tubes. Using the prism polarized white light was coupled to the sample. After passing through another polarizing element (analyzer) the reflex beam was collected with photodetector array.

Transducer Surface Preparation

According to antigen-antibody reaction transducer surface is covered by specific antibody layer that provides selective binding of relative antigen from added liquid. However, previous surface preparation leads to increasing of biosensor sensitivity level.

For biological material immobilization on the gold surface polyelectrolytes are widely used [Starodub et al., 2004]. Thin films obtained using small charged organic molecules are common since this molecules form insoluble polymer which electrostatically sorbs molecules with opposite charge [Starodub et al., 2001(b)]. In particular polyallylamin hydrochloride (PAA) immobilization on the transducer surface provides the acquisition of positive charge.

Also when biological molecules immobilization on the clean or previously treated by polyelectrolytes gold surface is provided using common physical adsorption [Starodub et al., 2001(a, b)], it leads to blocking of some active sites (antigen-binding sites) because of their interaction with gold surface. For overcoming this difficulty, different approaches can be used and one of them is protein A from *Staphylococcus aureus* previous immobilization [Starodub et al., 2001(b)]. The reason is that this protein has affinity to Fc-fragments of immunoglobulin G (IgG) and can bind its molecules without active sites participation. High quantity of free antigen-binding sites improves antigen binding and provides increasing of biosensor sensitivity.

Then surface was covered with protein A from *Staphylococcus aureus*. Solution of protein A was prepared using Tris-HCl buffer (pH 7.4). Concentration of solution was 1 mg/ml. After protein immobilization and all next stages of experiment transducer surface was washed with 0.9% saline solution.

After protein immobilization transducer surface was covered with *Salmonella typhimurium* specific polyclonal antibodies (Ab), its solution had concentration 1 mg/ml and it was prepared using Tris-HCl buffer (pH 7.4). Antibodies used at the experiment were provided by State scientific-research control institute of veterinary preparations and feed

additives of Ukraine. After Ab transducer surface was covered with bovine serum albumin (BSA) for blocking free non-specific binding centers on the gold surface. BSA powder was provided by Sigma (USA). BSA solution had concentration 1 mg/ml and it was prepared using Tris-HCl buffer (pH 7.4).

It has been observed that surface treatment using BSA after Ab immobilization did not make any contribution to the refraction angle quantity. It meant that there were no blanks on the gold surface and Ab concentration was enough for the formation of dense layer.

For detection using Spreeta, the model diluted solution of *S. typhimurium* with the number of concentrations (from 10^3 to 10^8 cells/ml) was prepared using 0,05 M tris-HCl buffer (pH 7,3). The time of the sample incubation with the transducer surface was about 5 min and after that it was washed by the above mentioned buffer.

For analysis using "Plasmonotest" model solution was prepared using 0.9% saline solution and dilution range of *S. typhimurium* was from 10^1 to 10^6 cells/ml. The time of the sample incubation with the transducer surface was about 10 min and after that the last was washed by the saline solution.

Transducer surface for TIRE based device was prepared in the same way as for SPR sensors. Range of dilution of initial *S. typhimurium* solutions was made in such way that 10 ml contained 1 and more cells (up to 10^5). The time of the sample incubation with the transducer surface was about 15 min. It was observed that the received value almost did not change after 30 min incubation. Maximal level of sensitivity was on the level of several cells in 10 ml (up to the fact that less than 5 cells).

Experimental Results and Discussion

In the case of Spreeta based biosensor, it has been defined that device sensitivity was on the level 10^3 – 10^7 cells/ml. Son and others had also used Spreeta for detection of *Salmonella enteritidis*. In their article, they reported that anti-Salmonella antibodies has been immobilized on the gold sensor surface using neutravidin. The sensor sensitivity they had was on the level 10^5 cfu/ml [Son et al., 2007]. Compared with previous results, it can be concluded that sensor sensitivity depends on transducer surface preparation method and can be noticeably improved using application of biological layers as described above. The diagram of the obtained results is presented in Fig.

2 (the changes of microorganism concentrations are indicated by the pointers above and the start of washing-by ones below).

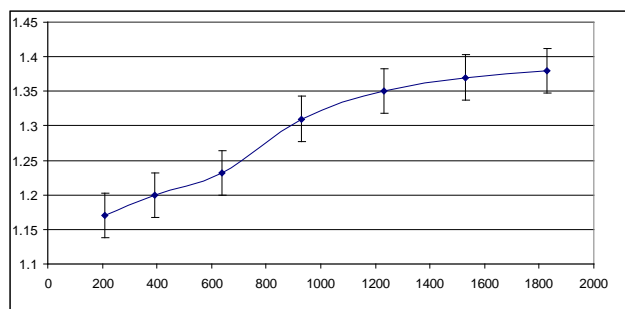


FIG. 2 SENSOR DIAGRAM OF *S. TYPHIMURIUM* SOLUTIONS ANALYSIS: SALINE SOLUTION – 10^3 – 10^8 CELLS/ML. ABSCISSA – TIME (SEC) AND ORDINATE – CHANGE OF RESONANT ANGLE

“Plasmonotest” detection level was within 10^1 – 10^6 cells/ml but statistically sufficient difference of results lays in ranges 10^2 – 10^6 cells/ml. It's higher than that in Spreeta case but it is not sufficient for all practice situations. Obtained results are shown in Fig. 3.

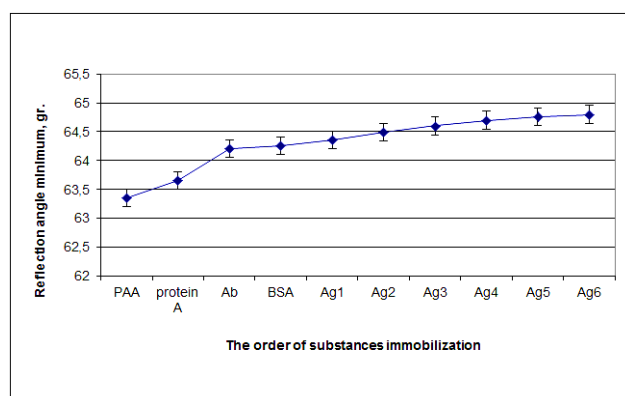


FIG. 3 DIAGRAM OF *S. TYPHIMURIUM* SOLUTIONS ANALYSIS USING “PLASMONOTEST” (AG – ROW OF *S. TYPHIMURIUM* SOLUTIONS FROM 10 – 1×10^6 CELLS/ML)

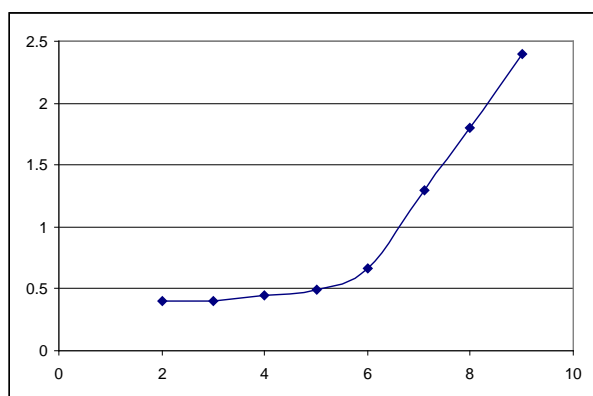


FIG. 4 DETERMINATION OF CONCENTRATION OF *SALMONELLA TYPHIMURIUM* IN CULTURE LIQUID USING ELISA METHOD. ABSCISSA – *S. TYPHIMURIUM* SOLUTION IN CONCENTRATIONS FROM 2×10^4 – 10^5 CELLS/ML; ORDINATE – OPTICAL DENSITY (E 492)

The sensitivity of above-mentioned immune sensors

was compared with that of the standard ELISA method, but the rate of analysis using the immune sensor was much faster. In case of the use of the ELISA method, the overall time of analysis may be up to 6 hours. For determination of *S. typhimurium* cells, the ELISA method provided sensitivity less than 10^4 cells/ml (Fig. 4). The analysis with this method was fulfilled by standard way [Prusak-Sochaczewski et al., 1989; Mansfield et al., 2000].

Biosensor based on the TIRE has shown higher sensitivity than the SPR based. Maximal level of sensitivity was on the level of several cells (less than 5) in 10 ml .

Sensitivity of the immune biosensor analysis does not strongly depend on way of the preliminary transducer surface treatment excluding situation where intermediate layer is absolutely absent. In our case, with the TIRE based immune biosensor, the signal decreased in such way that the sensitivity level was up to two orders less, than if the intermediate layer from PAA and protein A was created. In case of formation of such layer from protein A only, the above-mentioned sensitivity was less about one order than that if both components (PAA and protein A) were used. It was considered that this situation is connected with the blocking of the antigen-binding centers from one site and with the increasing of density of Ab immobilization on the transducer surface from other ones. As it is well known that the protein A has up to five centers for Ab binding and blocking some of them did not prevent that remained to interact with Fc fragments of Ab. PAA, maybe provide a high density of protein A immobilization on the electrostatic transducer surface.

TABLE 1 COMPARISON OF RESULTS OF *S. TYPHIMURIUM* DETERMINATION IN SOLUTIONS OBTAINED BY OPTICAL IMMUNE BIOSENSORS

Type of immune biosensor	Limit determination
“Spreeta” module, intermediate layers – PAA, protein A [Starodub et al., 2011; 2010]	10^3 – 10^7 cells ml ⁻¹
“Spreeta” module, intermediate layers – PAA, protein G [Starodub et al., 2011; 2010]	2×10^2 – 10^3 cells ml ⁻¹
“Spreeta” module, intermediate layers – dodecanthiol, protein A [Starodub et al., 2011; 2010]	5×10^3 – 10^4 cells ml ⁻¹
“Plasmonotest”, intermediate layers – PAA, protein A [Starodub et al., 2011; 2010]	10^1 cells ml ⁻¹
TIRE, intermediate layers – PAA, protein A [Starodub et al., 2011; 2010]	5 cells 10 ml ⁻¹
“Biacore”, intermediate layer – developed dextran [Gertie et al., 2003]	1.7×10^3 CFU ml ⁻¹
SPR, direct physical adsorption [Koubova et al., 2001]	10^6 cells ml ⁻¹
SPR, intermediate layer – mercapto-undecanoic acid, protein G [Oh et al., 2004]	10^2 cells ml ⁻¹

However, infection dose of pathogenic microorganisms is less than 10 cells per 100 ml [Ivnitski et al., 1999] therefore, it is necessary to find ways for the sensitivity increasing. Perhaps, satisfactory result can be obtained using monoclonal antibodies. In addition, it is necessary to pay attention to the effective methods of previous food samples preparation especially to the methods of bacteria cells concentration in the solution which will be examined next.

The obtained results and their comparison with existing literature data obtained by similar type immune biosensors are presented in Table 1.

Conclusions

Traditional cultural based methods are still the most widely used detection techniques and remain the gold standard for the detection of *Salmonella* due to their selectivity and sensitivity [Odumeru et al., 2012]. However, depending on the approach, standard culture methods typically require 5–7 days to obtain a result as they rely on the ability of *Salmonella* to multiply to visible colonies, which can then be characterized by performing additional biochemical and serological tests. More rapid immunological and molecular screening methods of detection have been devised to detect cell surface markers and nucleic acids, respectively. In most cases, these methods are high specific and sensitive but only a few of them have found wide use beyond the laboratories walls. The reason is that they are still many-stage, time consuming and expensive.

Our researches are directed to develop rapid, sensitive and high-specific biosensor devices for *Salmonella typhimurium* detection based on the different approaches such as surface plasmon resonance (SPR) and total internal reflection ellipsometry (TIRE).

In the case of Spreeta based biosensor, it has been defined that device sensitivity was on the level 10^3 – 10^7 cells/ml. "Plasmonotest" sensitivity was within 10^1 – 10^6 cells/ml. The highest detection level has been obtained using TIRE based biosensor and it was on the level of several cells in 10 ml (up to the fact that less than 5 cells). TIRE based biosensor sensitivity is the closest for practice cases of bacteria detection without previous samples preparation. Additionally, the lack of sensitivity of SPR based biosensors can be improved by developing of new methods of analysis with monoclonal antibodies using and/or previous samples preparation for antigen concentrating.

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95-117; "Immune Biosensor Based on the ISFETs for the Express Determination of *Salmonella typhimurium*". J. Electroanalysis, 24, 3, 600-606, 2012; "Efficiency of immune biosensor based on total internal reflection ellipsometry at the determination of *Salmonella*". Abstract of Chemical Sensors, May 21, 2012, DOI 10.5162/IMCS2012/P1.1.24. She worked as a Professor Assistant on the Department of Molecular Biology, Microbiology and biosafety in 2011.



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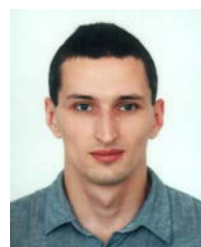


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He has more than 40 scientific publications including:

"Complex study of thin-film structures by optical methods and anodization spectroscopy". 7-th International Conference

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